Regulation of N-cadherin-mediated adhesion by the p35–Cdk5 kinase

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Background: The p35–Cdk5 kinase has been implicated in a variety of functions in the central nervous system (CNS), including axon outgrowth, axon guidance, fasciculation, and neuronal migration during cortical development. In *p35*$^{-/-}$ mice, embryonic cortical neurons are unable to migrate past their predecessors, leading to an inversion of cortical layers in the adult cortex.

Results: In order to identify molecules important for p35–Cdk5-dependent function in the cortex, we screened for p35-interacting proteins using the two-hybrid system. In this study, we report the identification of a novel interaction between p35 and the versatile cell adhesion signaling molecule β-catenin. The p35 and β-catenin proteins interacted *in vitro* and colocalized in transfected COS cells. In addition, the p35–Cdk5 kinase was associated with a β-catenin–N-cadherin complex in the cortex. In N-cadherin-mediated aggregation assays, inhibition of Cdk5 kinase activity using the Cdk5 inhibitor roscovitine led to the formation of larger aggregates of embryonic cortical neurons. This finding was recapitulated in *p35*$^{-/-}$ cortical neurons, which aggregated to a greater degree than wild-type neurons. In addition, introduction of active p35–Cdk5 kinase into COS cells led to a decreased β-catenin–N-cadherin interaction and loss of cell adhesion.

Conclusions: The association between p35–Cdk5 and an N-cadherin adhesion complex in the cortex, and the ability of p35–Cdk5 to regulate N-cadherin-mediated adhesion *in vitro*, suggest that p35–Cdk5 may play a role in the regulation of N-cadherin-mediated adhesion in cortical neurons.

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Background

The formation of the mammalian cerebral cortex requires the coordination of numerous events during embryonic development. Its complexity is illustrated by the large number of defects which have been identified in humans as lissencephalic disorders and in mice as cortical lamination defects. In humans, genetic mutations in Lis1, doublecortin, and filamin1 are responsible for the disease states type I lissencephaly, double cortex, and X-linked periventricular heterotopia, respectively [1]. In mice, cortical lamination errors have been identified in spontaneously occurring mouse strains and engineered deletion strains [2].

The phenotypes of the most extensively studied cortical lamination mouse model, *reeler*, are also found in *scrambler* mice, which along with the *mDab1*$^{-/-}$ and *yotari* strains have mutations in *mDab1* [3–7]. Molecular evidence that reelin, the protein defective in *reeler* mice, regulates mDab1 stability, and the identical phenotypes seen in mice mutated in *reelin* or *mDab1*, suggest that these molecules function in a linear signaling pathway [7,8]. In addition, mice lacking two low density lipoprotein receptors exhibit similar cortical phenotypes, suggesting that these receptors may form an additional component of a reelin signaling pathway [9].

Mice lacking p35–Cdk5 kinase activity, from genetic disruption of *p35* or *Cdk5*, also exhibit defects in the structure of the cortex [10,11]. The cortex in *p35*$^{-/-}$ or *Cdk5*$^{-/-}$ mice has distinct features from those in *reeler*-like mice, suggesting that the p35–Cdk5 kinase may be a component of an unrelated signaling pathway. In addition, phenotypes external to the cortex differ widely between *p35*$^{-/-}$, *Cdk5*$^{-/-}$, and *reeler*-like mice, suggesting that exclusively linear relationships are not likely to occur between p35–Cdk5 and reelin-dependent signaling pathways in the cortex. Based on the localization of p35–Cdk5 in cortical neurons [12] and its known function and substrates in the central nervous system [13,14], the hypothesis has been put forth that the p35–Cdk5 kinase functions to promote migration in cortical neurons by acting positively in pro-migratory signaling pathways and possibly antagonizing anti-migratory signals. From this hypothesis, we infer that the loss of p35–Cdk5 activity renders cortical neurons unable to migrate past their predecessors, leading to the inversion of neurons in the cortex of *p35*$^{-/-}$ mice [15].

One family of homophilic cell adhesion molecules, the cadherins, promotes adhesiveness by mediating adhesion in a calcium-dependent manner [16,17]. The cadherins have an inherent specificity for adhering to cells expressing the same subtype in a large family with over 20 members. Loss of cadherin-mediated adhesion has been implicated in promoting migration in several systems. In several tumor types, loss of E-cadherin-mediated adhesion is associated with the acquisition of invasive, metastatic characteristics by cancer cells [18,19].

In several neuronal systems, loss of N-cadherin expression is associated with the migration of neural cells. In the adult songbird forebrain, neurogenesis occurs from the ependymal/subependymal zone (SZ) precursor cells, which migrate into the brain parenchyma, where they mature [20]. SZ precursor cells express N-cadherin, but migrating cells of the same cohort lack N-cadherin [21]. Furthermore, suppression of N-cadherin activity facilitates cell body outgrowth from SZ explants, suggesting that down-regulation of N-cadherin is necessary for migration. In the neural crest, an analogous situation arises. Neural crest cells down-regulate N-cadherin and cadherin 6B while migrating [22–24] and re-express N-cadherin upon reaching their target [22,25]. When N-cadherin is overexpressed in neural crest cells, these cells fail to migrate [24], suggesting that down-regulation of cadherin expression is a prerequisite for migration. These studies, along with an abundance of evidence in tumor models, suggest that cadherins can act as anti-migratory proteins because of their adhesiveness and that the loss of cadherin-mediated adhesion facilitates cell migration.

In the developing cortex, N-cadherin is highly expressed in the ventricular zone and the cortical plate [26], where newly born post-mitotic cortical neurons begin and finish their migration, respectively. N-cadherin mRNA is not readily apparent in the intermediate zone, which cortical neurons traverse as they migrate to the cortical plate. N-cadherin expression diminishes rapidly in the early postnatal stages, when the majority of cortical migration is complete. Thus, N-cadherin is spatially and temporally expressed in the cortex in a manner consistent with a role in migration of cortical neurons.

In order to determine how p35–Cdk5 functions in the cortex, we took the approach of identifying p35-interacting proteins. In this manuscript, we report the identification of β-catenin as a p35-interacting protein. Complexes of p35 and Cdk5 contain β-catenin and N-cadherin, suggesting that p35–Cdk5 may regulate cell–cell adhesion. We provide evidence that loss of p35–Cdk5 leads to increased cadherin-mediated adhesion. From these results, we hypothesize that the p35–Cdk5 kinase functions to dynamically regulate cell adhesion during migration. Thus, the kinase may act to modulate the assembly of cadherin-based adhesion contacts during neuronal migration to allow neurons to migrate past their predecessors.

Results

β-catenin interacts with p35 in a two-hybrid screen and *in vitro*

In order to identify p35-interacting proteins by the two-hybrid system, an embryonic mouse cDNA library was screened with full-length p35 fused to the GAL4 DNA-binding domain as bait. Of approximately 1.4×10^6 total transformants, 120 clones were considered positive by interaction criteria (*URA3*-positive, *HIS3* selection, β-galactosidase-positive). Of these 120 positive clones, 15 encoded fragments of β-catenin. The library β-catenin cDNA clone used (Δ4 arm) began with the fifth armadillo (arm) repeat of β-catenin. Amino-terminal deletion fragments of β-catenin were generated and tested for interaction with p35 in the two-hybrid assay (Figure 1a). A deletion construct beginning at the sixth arm repeat (Δ5 arm) interacts strongly with p35, but further truncation (Δ6, Δ7 arm) leads to loss of interaction with p35, which suggests that the fifth and sixth arm repeats are necessary for interaction for p35.

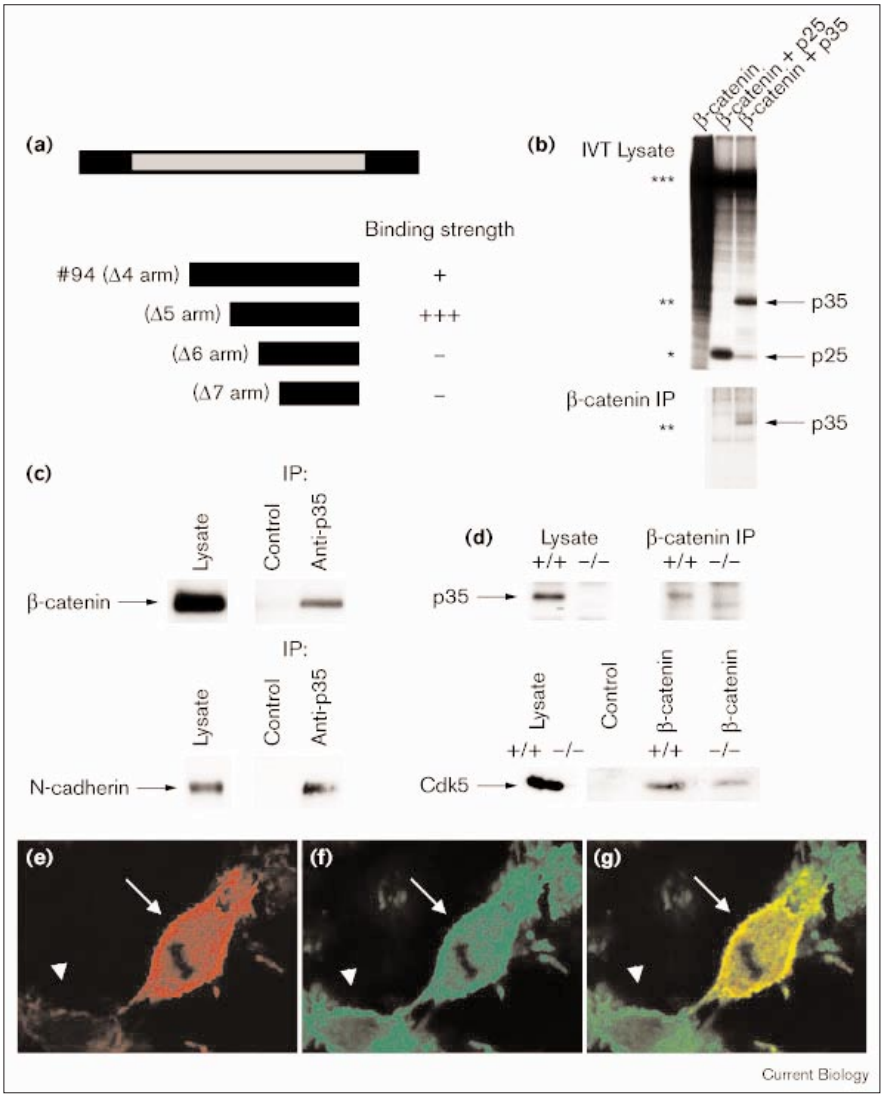
In order to detect a biochemical interaction *in vitro*, β-catenin, p35, and p25 constructs were transcribed and translated *in vitro* with ^{35}S-methionine. The p25 protein is a cleavage product of p35 that activates Cdk5 kinase robustly but lacks the amino-terminal 100 residues of p35 necessary for membrane targeting [27]. Upon immunoprecipitation using an anti-β-catenin antibody, labeled p35 was detectable, whereas p25 failed to immunoprecipitate efficiently (Figure 1b). This result suggests that the amino-terminal 100 residues of p35 are necessary for its association with β-catenin.

β-catenin and N-cadherin associate with p35 by co-immunoprecipitation

The two-hybrid interaction between p35 and β-catenin complemented studies that identified β-catenin associated with p35–Cdk5 in embryonic cortical neurons. Primary rat embryonic cortical neurons (removed at embryonic day 18, E18) were metabolically labeled with ^{35}S-methionine after 3 days in culture, and protein extracts were immunoprecipitated with control and affinity-purified anti-p35 antibodies. Major polypeptides of ~95 kDa and 135 kDa co-precipitated with p35 using anti-p35 antibody, but not with a control antibody (data not shown). In addition, two minor species of approximately 100 and 110 kDa were also detected. The intensity of the 100 and 110 kDa bands varied, but always appeared less intense than the 95 kDa and 135 kDa polypeptides (data not shown).

On the basis of the molecular sizes of 95 kDa and 135 kDa, we hypothesized that these bands corresponded to the proteins β-catenin and N-cadherin, respectively. Immunoprecipitation and immunoblotting experiments

Figure 1



The cell–cell adhesion signaling molecule β-catenin interacts with p35. **(a)** β-catenin was identified as a p35-interacting protein by screening an embryonic mouse cDNA library by the two hybrid system using full-length p35 as bait. The partial library clone used (#94) begins at the fifth armadillo repeat (Δ4 arm). Further deletion of one additional arm repeat (Δ5 arm) results in stronger binding, as measured by liquid β-galactosidase assay, but further deletion of two additional arm repeats (Δ6, Δ7 arm) results in complete loss of binding activity. **(b)** β-catenin, p25 and p35 constructs were transcribed and translated *in vitro* and immunoprecipitated using an anti-β-catenin antibody. Wild-type and two mutant versions of p35 (**) immunoprecipitated (IP) with β-catenin (***), whereas p25 (*), which lacks the amino-terminal region, failed to immunoprecipitate. **(c,d)** The p35–Cdk5 kinase associates with a β-catenin–N-cadherin complex in embryonic cortical neurons. (c) β-catenin western blot after immunoprecipitation with control or anti-p35 antibodies reveals an association between p35 and β-catenin. An N-cadherin western blot (bottom) indicates that it also co-immunoprecipitated with p35, presumably through its interaction with β-catenin. (d) Using an anti-β-catenin antibody, p35 co-immunoprecipitated with β-catenin from wild-type but not from *p35*$^{-/-}$ embryonic cortical extracts (top). Cdk5 also co-immunoprecipitated with β-catenin from wild-type extracts (bottom), indicating that the p35–Cdk5 complex is associated with β-catenin. Notably, Cdk5 also co-immunoprecipitated with β-catenin from *p35*$^{-/-}$ brain lysates, indicating that other p35 family members, such as p39, may connect Cdk5 to β-catenin. **(e–g)** Confocal imaging reveals that p35 colocalizes with β-catenin in COS cells infected with a p35 adenovirus (arrow). (e) p35 strongly localizes to the cell periphery (Texas Red), as does (f) β-catenin (fluorescein isothiocyanate, FITC, green). (g) In the merged image, p35 and β-catenin colocalize significantly at the cell periphery. Arrowhead marks an uninfected cell.

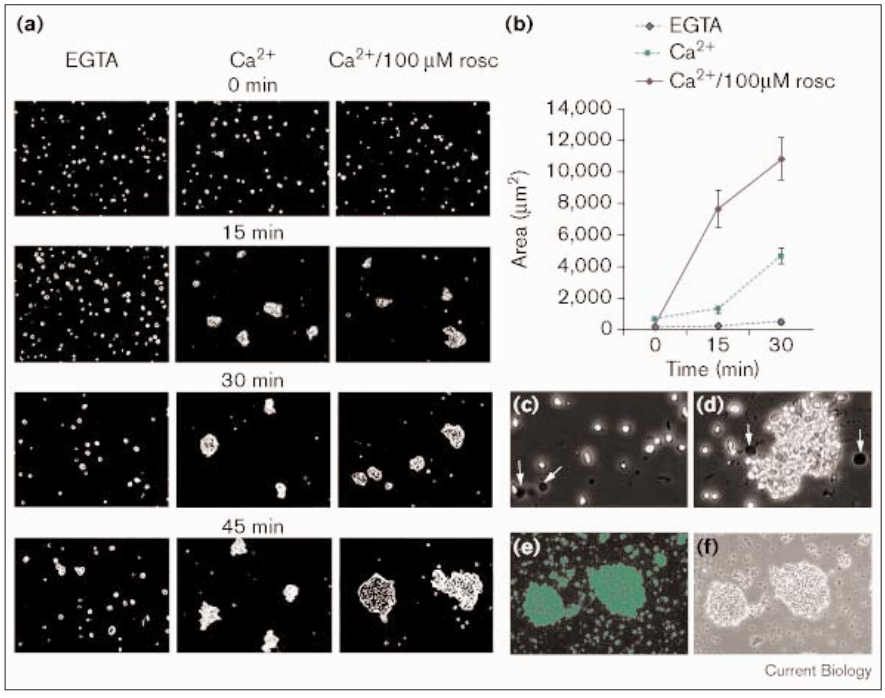
from embryonic cortex extracts revealed that β-catenin co-immunoprecipitated with p35 (Figure 1c). In addition, N-cadherin co-immunoprecipitated with p35 in embryonic cortical neurons (Figure 1c). Conversely, p35 was detected in β-catenin immunoprecipitates from embryonic cortical lysates prepared from wild-type but not from *p35* knockout mice (Figure 1d). Cdk5 was also present in β-catenin immunoprecipitates of wild-type embryonic cortical neurons (Figure 1d), indicating that not only p35 but also its kinase subunit Cdk5 was present in a β-catenin complex. Similar interaction results were obtained from transfected COS cells (data not shown).

Interestingly, Cdk5 could also be co-immunoprecipitated with β-catenin from cortical lysates of *p35* knockout mice (Figure 1d). This finding is consistent with our observation that the p35 family member p39, which also binds to and activates Cdk5, (S. Humbert and L-H. Tsai, unpublished observations), also associates with β-catenin. Hence, in the *p35*$^{-/-}$ background, Cdk5 is likely to associate with β-catenin through p39.

p35 and β-catenin colocalize in transfected COS cells

As we detected an interaction between p35 and β-catenin, we were interested in determining whether p35 and

Figure 2



Aggregation of rat embryonic cortical neurons. **(a)** Dissociated embryonic cortical neurons were aggregated in the presence of 1 mM EGTA, 1 mM $CaCl_2$, or 1 mM $CaCl_2$ with 100 μM roscovitine (rosc). After 0, 15, 30, and 45 min, aggregates were fixed and viewed using phase contrast microscopy. Aggregates formed in the presence of 1 mM $CaCl_2$ but not 1 mM EGTA. In the presence of 1 mM $CaCl_2$ and 100 μM roscovitine, aggregates were larger. **(b)** Quantification of aggregation in rat cortical neurons. In the presence of 1 mM $CaCl_2$, aggregates formed over the time course, whereas aggregates failed to form in the presence of 1 mM EGTA. In the presence of 1 mM $CaCl_2$ and 100 μM roscovitine, aggregates were twofold larger. At each time point for the different conditions, aggregates were plated onto poly-D-lysine-coated coverslips and fixed. Surface area of 10 aggregates (per time point per condition) was measured using Image Pro Plus imaging software and averaged. Bars represent standard error. **(c,d)** EGTA and roscovitine do not perturb cell viability in the aggregation assay. E18 rat primary cortical neurons were subjected to aggregation for 30 min in the presence of either (c) 1 mM EGTA or (d) 1 mM Ca^{2+} and 100 μM roscovitine. Cell viability was assessed by trypan blue staining (0.1%). In both cases, the vast majority of neurons were healthy (white) and only a very few scattered cells underwent cell death (black, arrows). Importantly, cell death did not occur in the neuronal aggregates. **(e,f)** Staining of aggregates using the TuJ1 antibody, which recognizes neuron-specific β3-tubulin, indicates that the aggregates were composed mainly of neurons. (e) Immunofluorescence; (f) phase contrast view.

β-catenin colocalize in COS cells. COS cells infected with a p35-expressing adenovirus were fixed and immunostained using anti-p35 and anti-β-catenin antibodies (Figure 1e–g). Confocal microscopy revealed that p35 (Figure 1e) and endogenous β-catenin (Figure 1f) strongly colocalized at the cell periphery (Figure 1g), indicating that the p35–Cdk5 kinase and β-catenin are in close proximity at cadherin adhesion contacts.

Calcium-stimulated aggregation in embryonic cortical neurons is dependent on N-cadherin

Interactions between the p35–Cdk5 kinase, β-catenin, and N-cadherin suggested that the kinase may regulate cadherin-mediated cell–cell adhesion in embryonic cortical neurons. To test whether the p35–Cdk5 kinase could modulate cadherin-mediated adhesion, calcium-stimulated neuronal aggregation assays were performed using primary embryonic neurons from rat cortices.

Dissociated cortical neurons prepared from cortices of E17–19 rat embryos were aggregated by rotation (80 rpm, 37°C; [28]). Aggregates formed in the presence of 1 mM $CaCl_2$ but not in the presence of 1 mM EGTA (Figure 2a; middle and left columns, respectively). Quantification of the two-dimensional surface area of individual aggregates revealed that calcium led to increased aggregation whereas EGTA led to negligible aggregate formation (Figure 2b).

It was formally possible that the lack of aggregate formation in the presence of EGTA resulted from diminished cell viability. To address this issue, cortical neurons were stained with trypan blue (0.1%) immediately after plating as well as after completion of rotation. As depicted in Figure 2c, cortical neurons were readily viable throughout the aggregation procedure, indicating that lack of aggregation was indeed caused by sequestration of calcium. To determine whether aggregates were composed predominantly of neurons, the aggregates were immunostained for the neuron-specific tubulin isoform β3-tubulin (TuJ1 antibody; [29]). Aggregates stained robustly with TuJ1 (Figure 2e,f), with few glial fibrillary acidic protein (GFAP)-positive cells (data not shown), indicating that the aggregates were composed mainly of cortical neurons.

We also examined β-catenin and N-cadherin protein levels from extracts prepared during aggregation (Figure 3a). In the presence of calcium, β-catenin and N-cadherin protein levels were stable throughout the course of the experiment (30 min). In the presence of EGTA, however, N-cadherin protein levels rapidly diminished while β-catenin levels

remained constant. The loss of N-cadherin protein in the absence of calcium is consistent with previous studies indicating that the loss of calcium renders the cadherin sensitive to cleavage by proteases [16].

In order to determine whether the calcium-stimulated aggregation was mediated specifically by N-cadherin, aggregation experiments were performed in the presence of 1 mM N-cadherin-blocking peptide [30,31]. This peptide specifically inhibited aggregate formation (Figure 3c), whereas a scrambled peptide failed to do so (Figure 3b). These results suggest that calcium-stimulated aggregate formation of cortical neurons requires N-cadherin function. Together, these experiments provide compelling evidence that N-cadherin-mediated adhesion serves as an important mechanism for embryonic cortical neurons to adhere to each other during cortical development.

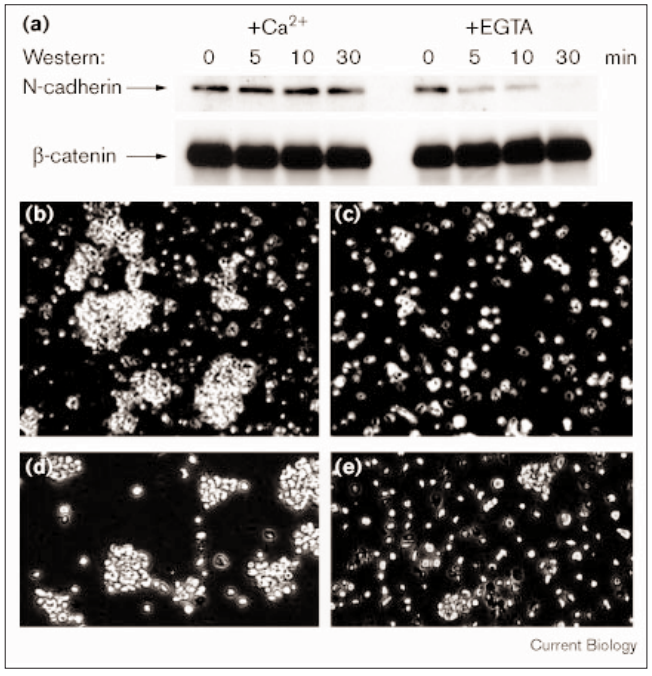
Inhibition of the Cdk5 kinase increases calcium-stimulated neuronal aggregation

In order to determine the role of Cdk5 in this system, the Cdk5 inhibitor roscovitine [32] was added to the culture medium upon initiation. Roscovitine was applied to the cortical neurons prior to calcium-stimulated aggregation. In the presence of calcium and 100 μM roscovitine (Figure 2a, right column; Figure 2b), a two-fold increase in aggregate size was observed, suggesting that inhibition of Cdk5 allowed larger aggregates to form. Aggregate formation in the presence of EGTA and roscovitine (50 and 100 μM) was negligible (data not shown), suggesting that roscovitine itself does not induce aggregation. To verify that roscovitine treatment did not induce cell death, cortical neurons were again subjected to trypan blue staining at the end of rotation. Roscovitine treatment alone did not affect cell viability (data not shown). More importantly, however, cell viability was also preserved in the neuronal aggregates that formed in the presence of calcium and roscovitine (Figure 2d). Later observation confirmed that the increased aggregate size obtained in the presence of calcium and roscovitine was not merely due to clumping of dying cells.

So far, all aggregation assays were conducted with primary cortical neurons from rats. In order to determine more precisely whether the loss of p35–Cdk5 kinase activity led to an increase in aggregate formation, aggregation assays were performed with primary neurons from wild-type and *p35*$^{-/-}$ mouse cortices (Figure 4a,b). Postnatal day 0 (P0) cortical neurons were prepared simultaneously from wild-type and *p35*$^{-/-}$ mice using timed pregnant homozygous wild-type and *p35*$^{-/-}$ mouse crosses. Aggregation using wild-type cortical neurons exhibited properties of cadherin-mediated aggregation, and calcium-stimulated aggregation was increased in the presence of calcium and 50 μM roscovitine (Figure 4a). Aggregation using *p35*$^{-/-}$ cortical neurons was dependent on calcium, but the size of aggregates was equivalent to those of wild-type in the presence of calcium and roscovitine (Figure 4b). Furthermore, the presence of roscovitine did not increase the ability of *p35*$^{-/-}$ cortical neurons to aggregate. These results indicated that loss of the p35–Cdk5 kinase activity resulted in an increase in the ability of cortical neurons to aggregate in a calcium-dependent manner.

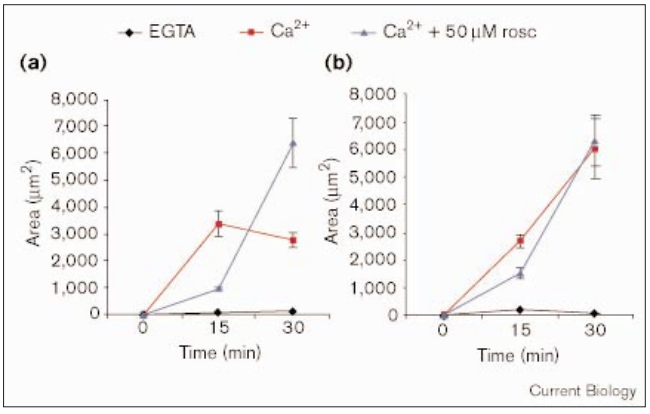
The results from the aggregation assay imply that the increased adhesiveness of neurons that are compromised in Cdk5 function is mediated by N-cadherin. To address this point directly, it was necessary to determine whether

Figure 3



N-cadherin is a central mediator of cellular aggregation in the neuronal aggregation assay. **(a)** β-catenin and N-cadherin levels during aggregation. Protein extracts were prepared from embryonic cortical neurons 0, 5, 10, and 30 min after the initiation of aggregation in the presence of EGTA or $CaCl_2$. Blots were probed for β-catenin and N-cadherin. In the presence of EGTA, N-cadherin protein levels diminished rapidly, but β-catenin protein levels remained unchanged. In the presence of $CaCl_2$, β-catenin and N-cadherin levels remained stable. **(b,c)** N-cadherin-blocking peptide disrupts calcium-dependent aggregation of primary cortical neurons. In order to determine whether calcium-stimulated aggregation is dependent on cadherins, (c) N-cadherin-blocking peptide (17-mer) or (b) a scrambled peptide (17-mer) was added to the aggregation reaction of rat primary cortical neurons. N-cadherin-blocking peptide, but not scrambled peptide, disrupted aggregate formation significantly. **(d,e)** N-cadherin-blocking peptide diminishes aggregation of roscovitine-treated primary cortical neurons. E18 rat primary cortical neurons were pretreated with either (d) scrambled peptide (control) or (e) N-cadherin-blocking peptide and then induced to aggregate in the presence of 1 mM calcium and 100 μM roscovitine. Neurons treated with N-cadherin-blocking peptide showed a significant reduction of aggregate size in comparison to neurons treated with scrambled peptide.

Figure 4



Quantification of aggregation in mouse cortical neurons. **(a,b)** Aggregation assay performed in mouse wild-type and *p35*$^{-/-}$ cortical neurons. P0 cortical neurons were prepared from newborn wild-type and *p35*$^{-/-}$ mice. (a) Wild-type neurons aggregated in the presence of 1 mM $CaCl_2$, and exhibited larger aggregates in the presence of 50 μM roscovitine and 1 mM $CaCl_2$, whereas aggregates failed to form with 1 mM EGTA. The properties of aggregation are consistent with a cadherin-mediated mode of adhesion in these assays. (b) *p35*$^{-/-}$ neurons exhibited high aggregation in the presence of $CaCl_2$ and were refractory to roscovitine. At 30 min, aggregate sizes for *p35*$^{-/-}$ cortical neurons with $CaCl_2$ were similar to wild-type cortical neurons with $CaCl_2$ and roscovitine. Surface area of 10 aggregates (per time point per condition) was measured using Image Pro Plus imaging software and averaged. Bars represent standard error.

the N-cadherin-blocking peptide was able to block the enhanced aggregation caused by roscovitine or by loss of p35 function. To this end, cortical neurons were treated with roscovitine and subjected to either scrambled or N-cadherin-blocking peptide. Clearly, neurons that were incubated with blocking peptide formed significantly smaller aggregates (Figure 3e) than neurons that were treated with scrambled peptide (Figure 3d). Hence, not only was the basic aggregation property of cortical neurons dependent on N-cadherin, but also the increased aggregation of neurons with compromised Cdk5 function relied, at least in part, on N-cadherin function.

p35 destabilizes β-catenin–N-cadherin interactions in COS cells

Increased aggregation in embryonic cortical neurons upon Cdk5 inhibition suggests that the p35–Cdk5 kinase may dynamically modulate cadherin-mediated adhesion. To test whether the presence of p35–Cdk5 kinase activity leads to alterations in catenin–cadherin interactions and cell adhesion, COS cells, which endogenously express β-catenin, N-cadherin, and Cdk5 (Figure 5a), were transfected with increasing amounts of a mammalian expression vector driving *p35* cDNA expression. Lysates were prepared 48 hours after transfection and membrane fractions immunoprecipitated with an anti-β-catenin antibody. Western blots were probed for N-cadherin. Transfection of p35 led to a decrease in the amount of N-cadherin associated with β-catenin (Figure 5b, left panel). In contrast, a second glycine-to-alanine mutant of p35 (G2A p35), which is known to no longer associate with the cell membrane [27] did not perturb the β-catenin–N-cadherin association (Figure 5b, right panel). This result suggests that p35–Cdk5 can specifically destabilize β-catenin–N-cadherin interactions, as long as it is allowed to associate with the cell membrane. As it had been shown previously that the interaction between β-catenin and cadherins is essential for cadherin-mediated cell–cell adhesion [33,34], these results suggest that p35–Cdk5 may modulate cadherin-mediated adhesion by regulating the β-catenin–N-cadherin association.

Introduction of p35 into COS cells also had robust effects on cellular adhesion. Infection using a p35-expressing adenovirus led to subtle rounding up of COS cells (Figure 5d), whereas control vector-infected cells maintained normal adhesion (Figure 5c). Interestingly, infection using a p35–Cdk5-expressing virus led to robust rounding up and loss of adhesion in COS cells (Figure 5e). These results are consistent with the idea that activation of the p35–Cdk5 kinase can lead to loss of adhesion.

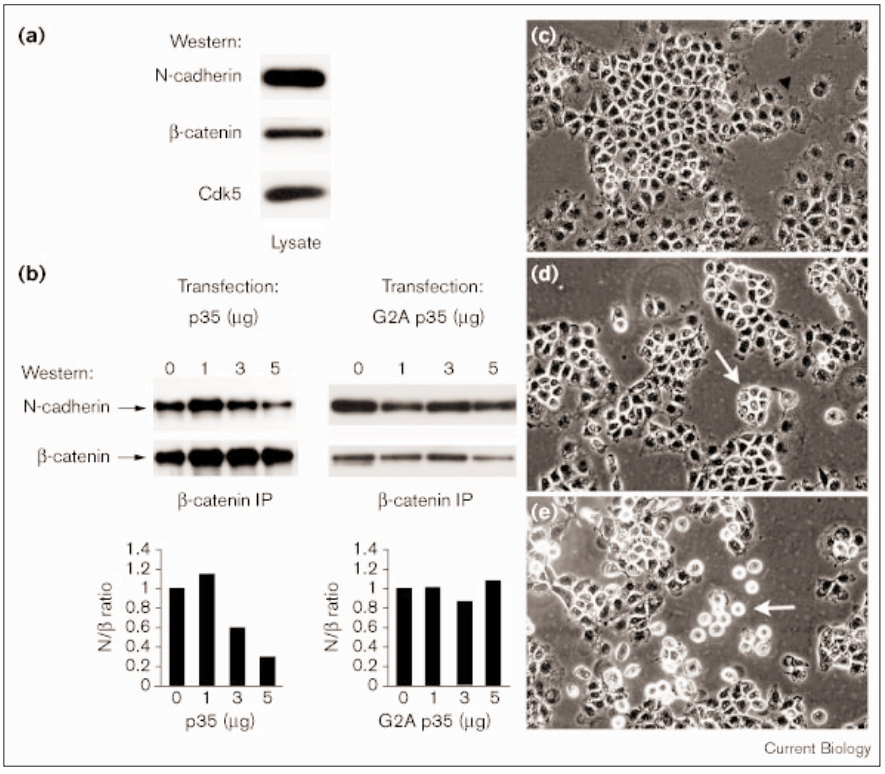
Discussion

In this study, we report the identification of the cell–cell adhesion signaling molecule β-catenin as a p35-interacting protein by two-hybrid screen (Figure 1a). The p35–Cdk5 kinase is associated with β-catenin and the cell–cell adhesion molecule N-cadherin (Figure 1c,d) in the embryonic and adult cortex. On the basis of the presence of other associated proteins in p35 immunoprecipitation experiments from embryonic cortical neurons (data not shown), it is likely that other components of the adherens junction complex, such as α-catenin, are also associated with the p35–Cdk5 kinase via the direct interaction between p35 and β-catenin. Using aggregation assays as a measure of N-cadherin-mediated adhesion, we have demonstrated that loss of Cdk5 kinase activity led to increased aggregation of cortical neurons (Figures 2–4). Furthermore, we have shown that introduction of p35–Cdk5 into COS cells led to a decreased β-catenin–N-cadherin interaction and loss of cellular adhesion. Cumulatively, these results suggest that p35–Cdk5 may function to modulate cadherin-mediated adhesion in cortical neurons.

N-cadherin-mediated aggregation in embryonic cortical neurons

The interaction between the p35–Cdk5 kinase and components of cadherin-mediated cell adhesion suggests that the p35–Cdk5 kinase may play a regulatory role in cadherin-mediated adhesion, and the co-localization of p35 and β-catenin at the membrane in COS cells further supports this notion (Figure 1e–g). To test this possibility, a neuronal aggregation assay using embryonic cortical

Figure 5



p35 modulates the β-catenin–N-cadherin association in COS cells. **(a)** Cdk5 is present in membrane lysates of COS cells. COS cells were lysed and membrane fractions prepared. Lysates of the membrane fraction were probed for Cdk5, β-catenin, and N-cadherin. **(b)** N-cadherin western blot after β-catenin immunoprecipitation (IP) indicates that transfection of p35 led to a decreased association between β-catenin and N-cadherin. Increasing amounts of wild-type or G2A mutant p35 (control) were transfected into COS cells. The association of β-catenin and N-cadherin was then assessed by western analysis of β-catenin and N-cadherin on β-catenin immunoprecipitates. With increasing amounts of p35 plasmid transfected (1, 3, 5 μg), wild-type p35 (left) but not the G2A mutant p35 (right) promoted dissociation of β-catenin from N-cadherin. Image quantification using NIH Image (graphs) reveals that the ratio of N-cadherin bound to β-catenin decreased with increasing amounts of p35 plasmid transfected into COS cells. **(c–e)** COS cells were infected with (c) control adenovirus or adenoviruses expressing (d) p35 or (e) p35 and Cdk5. (e) COS cells infected with p35–Cdk5 became rounded and lost adhesion (arrow), whereas (c) control infected cells remained attached to the substratum. (d) p35 infected cells exhibit a moderate phenotype. Arrow in (d) marks a cluster of rounded COS cells. Note the overall loss of membraneous structures in (d) p35 infected cells versus (c) control infected cells (arrowhead).

neurons was utilized. In this study, we found that calcium stimulates cellular aggregation of embryonic cortical neurons (Figure 2). We further confirmed that the cadherins, and more specifically, N-cadherin, is the central mediator of cellular aggregation in our assay using cortical neurons (Figure 3b–e). Not only does N-cadherin have a role in mediating the basic property of calcium-dependent aggregation (Figure 3b,c), but it also mediates, at least in part, the Cdk5-dependent modulation of adhesion in primary cortical neurons (Figure 3d,e). In fact, the N-cadherin-dependency of aggregation suggests that N-cadherin-mediated adhesion is likely to be an important mediator of neuron–neuron interaction, as TuJ1 staining revealed that the aggregates were formed predominantly of neurons (Figure 2e,f). This finding is consistent with the robust expression of N-cadherin in the ventricular zone and cortical plate [26], which together contain the vast majority of cortical neurons in the developing cortex. We surmise that N-cadherin-mediated aggregation in these assays reflects the cohesiveness of cortical plate neurons *in vivo*. Based on this assumption, the increase in aggregate size with Cdk5 kinase inhibition (Figures 2a,b and 4) may indicate that $p35^{-/-}$ cortical neurons maintain a greater degree of N-cadherin-mediated adhesion than their wild-type counterparts.

As loss of Cdk5 kinase activity by pharmacological inhibition or p35 gene knockout results in increased cell adhesion, we also attempted to evaluate the consequence of overexpression of p35–Cdk5 in cell adhesion. We found that overexpression of p35–Cdk5 caused COS cells to lose adhesion and round up (Figure 5c–e). In addition, increasing amounts of p35 expression caused a corresponding decrease in β-catenin–N-cadherin association (Figure 5b). These observations not only support the notion that p35–Cdk5 destabilizes N-cadherin mediated cell adhesion, but also suggest that the action of p35–Cdk5 is to generally regulate the assembly/disassembly of cell–cell adhesion complexes.

Models of p35–Cdk5 action on N-cadherin-mediated adhesion

Several models can be proposed to account for the regulation of cadherin-mediated adhesion by the p35–Cdk5

kinase. As p35–Cdk5 is a protein serine/threonine kinase, it may phosphorylate one or more components of the cadherin-adhesion complex, ultimately leading to decreased cell adhesion. Indeed, we found that β-catenin contains three minimal consensus sites for phosphorylation by Cdks. However, there is little evidence to support a role for serine/threonine phosphorylation as a modulator of cadherin-mediated adhesion in cortical neurons. On the other hand, evidence of regulation of tyrosine phosphorylation of β-catenin and the cadherins by the EGF receptor, the kinase Src, and the phosphatases PTP1B and LAR suggests that tyrosine phosphorylation may serve as an important mechanism to regulate cadherin-mediated adhesion [35–41]. The role of tyrosine phosphorylation in cadherin-mediated adhesion is interesting in light of the recent identification of a Cdk5-interacting protein, Cables, which bridges Cdk5 and the non-receptor tyrosine kinase Abl (L. Zukerberg, G. Patrick, M. Nicolic, S. Humbert, L. Lanier, F. Gertler, *et al.*, unpublished observations). Additionally, the p35–Cdk5 kinase may function as a scaffold to assemble molecules that act to destabilize N-cadherin-mediated adhesion. For instance, we have also shown previously that p35–Cdk5 interacts with the active form of the small GTPase Rac, and modulates Pak1 kinase activity [42]. As Rac activity is necessary for cadherin-mediated adhesion, it is possible that the p35–Cdk5 kinase may regulate cadherin-mediated adhesion by regulating a Rac-dependent signaling pathway.

On the basis of our immunoprecipitation and western blot analysis, only about 1% of endogenous β-catenin binds to p35, whereas more than 10% of total p35 binds to β-catenin in the embryonic brain lysates. This observation suggests that whereas the β-catenin–N-cadherin complex may be one of the major targets for the p35–Cdk5 kinase, only a small fraction of the β-catenin–N-cadherin complex in the developing cortex is actually regulated by p35–Cdk5. Indeed, we failed to detect a difference in the overall levels and association of β-catenin and N-cadherin in membrane extracts derived from the embryonic and adult cortices of *p35*$^{-/-}$ mice (data not shown). Thus, it is possible that p35–Cdk5 regulation of N-cadherin mediated adhesion is only crucial in a very specific population of migrating cortical neurons. In fact, it may be that p35–Cdk5 regulation of N-cadherin-mediated adhesion is relevant to neurons only when they traverse the intermediate zone of the developing cortex.

Cadherins in migration

The loss of cadherin-mediated adhesion has profound effects in deregulated disease states and developmental processes. In breast, prostate, and other cancers, loss of E-cadherin-mediated adhesion is associated with invasive, metastatic properties of cancer cells [18,19]. During neural crest migration, loss of N-cadherin-mediated adhesion seems to be a prerequisite for proper migration, as overexpression of N-cadherin in migrating neural crest cells completely inhibits their migration [24]. These examples highlight the importance of modulating cadherin-mediated adhesion in the development and maintenance of an organism.

In the embryonic cortex, the expression pattern of N-cadherin is consistent with it having a role in migration similar to one in the chick neural crest and avian forebrain. Interestingly, N-cadherin is highly expressed in the embryonic cortex during the period of corticogenesis and diminishes rapidly after this period [26]. We have previously shown that the migration behavior of the first cohort of postmitotic neurons in *p35*$^{-/-}$ mice is comparable to wild type, but that subsequently born neurons failed to penetrate their predecessors [15]. Thus, the *p35*$^{-/-}$ cortex develops in an inverted manner from the normal 'inside-out' pattern of cortical development. In light of this defect in *p35*$^{-/-}$ mice, it is temping to hypothesize that the p35–Cdk5 kinase prevents stable N-cadherin-mediated adhesion contacts from forming while cortical neurons migrate through the cerebral wall. This would allow migrating cortical neurons to move past their predecessors in the embryonic cortex. The loss of p35–Cdk5 kinase activity would hinder the ability of migrating cortical neurons to regulate cadherin-mediated adhesion, leading to the formation of stable cadherin adhesion complexes and inappropriate adhesion to previously generated cortical neurons. This inappropriate adhesion would cause a blockage of migration, preventing the migrating neurons to traverse through their predecessors. In this manner, the neuronal predecessors would be 'pushed' towards the cerebral surface, thus creating the inversion of the normal 'inside-out' migration pattern. It would be interesting to determine whether inhibition of N-cadherin mediated adhesion is able to reverse, at least in part, the *p35*$^{-/-}$ cortical lamination defect.

Finally, the regulation of N-cadherin mediated adhesion by p35–Cdk5 appears very interesting in light of a recent report [43] suggesting that members of the cadherin-related neuronal receptor (CNR) family have functional properties that identify them as receptors for reelin. Hence, these findings introduce the idea that cadherin-type adhesional molecules may play a significant role in regulating corticogenesis altogether. However, we feel that although N-cadherin and CNR family members are related molecules, their role in corticogenesis may be distinct. In fact, destabilization of N-cadherin may be important for the initial phase of migration, whereas CNR family members may mediate the reelin-dependent termination of migration. The following arguments may be considered in support of this notion. First, although N-cadherin and CNR family members are both expressed in the developing cortical plate, CNR family members are not expressed in the ventricular zone. Therefore, they cannot be involved in impeding the initial phase of migration, as we hypothesize

for N-cadherin. Second, the cytoplasmic domain of the CNR family members is quite distinct from that of the classical cadherins. In fact, this domain has been shown to specifically interact with the tyrosine kinase Fyn, which, in turn, has been implicated in mDab signaling ([43] and references therein). In this sense, the primary role of CNR family members may be to mediate the reelin signaling-pathway rather than to mediate homotypic adhesion. Consistent with this notion, the cytoplasmic domain of CNR family members does not harbor any region that resembles the beta-catenin interaction domain of classical cadherins. Third, the EC1 domain of CNR family members harbors a RGD motif, which is recognized by the integrin receptor family. This may underscore the importance of the CNR family members in heterotypic adhesion, such as the adhesion between glia and neurons. In fact, there have been numerous reports that point to the importance of integrins in glia–neuron interaction [44]. It will be interesting to see what direction further research will take in order to dismantle the various roles of cadherin-type molecules in the development of cortical layering.

Materials and methods

Two-hybrid screen

A modified two-hybrid system was used [45]. The bait plasmid was generated by cloning full-length p35 into the bait vector pPC97. Approximately 1.4×10^6 transformants of an embryonic mouse cDNA library fused to the GAL4 activation domain were screened using selection criteria (growth on HIS3, URA3, β-galactosidase assay).

In vitro *binding experiment*

Mammalian expression vectors expressing β-catenin (a gift of P. Polakis, Onyx Pharmaceuticals), p25, and p35 (T138A point mutant) were transcribed and translated *in vitro* with ^{35}S-methionine using the Promega TNT system. β-catenin and associated proteins were immunoprecipitated using anti-β-catenin antibody (Transduction Laboratories) and protein-G–Sepharose beads (Pharmacia).

Rat primary embryonic cortical cultures

Primary cultures of embryonic rat cortical neurons were prepared as described [12]. In brief, E17–19 pregnant rats were sacrificed by CO_2 inhalation and the embryos removed. Dissociated embryonic neurons were plated onto poly-D-lysine/laminin-coated plates at: $6–12 \times 10^6$ cells per plate (100 mm plate), $2–4 \times 10^6$ cells per plate (60 mm plate), $1–2 \times 10^6$ cells per well (6 well plate), $2–4 \times 10^5$ cells per well (24 well plate). For metabolic labeling, cell culture media was replaced with pre-warmed 3 ml DMEM (without methionine) supplemented with 600 μCi ^{35}S-methionine. After three hours, neurons were lysed in ELB lysis buffer (50 mM Tris–HCl pH 7.4, 250 mM NaCl, 0.1% NP-40, 1 mM EDTA, 1 mM dithiothreitol) with protease inhibitors on ice for 20 min. The lysates were cleared by centrifugation at 13,000 rpm.

Lysates, immunoprecipitations, and western blots

Lysates from embryonic and adult cortices were prepared by dounce homogenization in ELB lysis buffer, cleared by centrifugation, and quantitated using the Bradford assay. The rabbit polyclonal antibody raised against histidine-tagged (His_6)–p35 was affinity purified against bacterially produced glutathione-S-transferase-tagged (GST)–p25. The β-catenin antibody (Transduction Laboratories) was used at 1:1000 for western blots; 0.5–1 μg was used for immunoprecipitations. The N-cadherin antibody used for western blots (hybridoma supernatant, a gift from Margaret Wheelock) was diluted 1:50. Protein-A–Sepharose beads were used for p35 immunoprecipitations; protein-G–Sepharose beads were used for β-catenin immunoprecipitations.

Neuronal aggregation assay

Dissociated rat embryonic cortical neurons and wild-type or *p35*$^{-/-}$ P0 cortical neurons were washed in plating media and resuspended in plating media with 1 mM EGTA, 1 mM $CaCl_2$, and 1 mM $CaCl_2$ with roscovitine (50 or 100 μM). Cells (1×10^7) in 2 ml medium were aggregated in 6-well plates at 80 rpm in a 37°C floor shaker. At each time point, aggregates were transferred with a plastic pasteur pipet onto coverslips previously coated with poly-D-lysine. After 15 min at 37°C, aggregates were fixed with 4% paraformaldehyde (10 min) and permeabilized with PBS containing 0.2% Triton X-100 (10 min). Coverslips were rinsed with PBS followed by H_2O. Cresyl violet stain was applied for 5 min and the coverslips mounted with PBS containing 50% glycerol. The surface area of ten aggregates for each condition and time point was measured using Image Pro Plus imaging software. The N-cadherin 17-mer blocking peptide (ARFHLRAHAVDINGGNQV) and scrambled peptide (VAVLYEKSGIAYHNSAS) were used essentially as described [30,31]. In brief, peptides were used at a concentration of 1 mM and preincubated with the primary neurons for 30 min at room temperature, after which aggregation was induced as described above.

Immunostaining

COS cells infected with p35-expressing adenovirus were fixed in 4% paraformaldehyde for 10 min at room temperature. After washing with PBS + 0.2% Tween-20 (PBST), cells were permeabilized with PBS + 0.2% Triton X-100 and blocked in PBST + 10% goat serum. Affinity-purified anti-p35 antibody (1:1000) and monoclonal β-catenin antibody (1:1000) were applied in block solution. Texas Red conjugated anti-rabbit secondary antibody and FITC-conjugated anti-mouse secondary antibody were used. Aggregates were fixed in 4% paraformaldehyde as described and washed three times in PBS + 0.2% Tween-20; blocked for 1 h in PBST containing 10% goat serum and 0.1% Triton X-100, and incubated with TuJ1 antibody (1:500 dilution; Research Diagnostics) for 1–2 h; washed in PBST, and incubated with FITC-conjugated anti-mouse secondary antibody.

COS cell transfection and infection

COS cells were transfected in 100 mm plates using the calcium phosphate method. The mammalian expression plasmids were either wild-type p35 or the second glycine-to-alanine mutant G2A p35. The latter mutant was used as a specificity control because the mutation abolishes localization of p35 to the cell membrane. At 48 h after transfection, cells were lysed in STM lysis buffer for 20 min on ice. Extracts were spun at $600 \times g$ for 15 min at 4°C. The supernatant was considered a crude cytoplasm extract. The pellet was resuspended in STM lysis buffer and respun. After the spin, the pellet was resuspended in STM + 0.5% NP-40. The extract was respun ($600 \times g$ for 15 min at 4°C) and the resulting supernatant was considered crude membrane fraction. β-catenin immunoprecipitation was performed as described. Control adenovirus and viruses expressing p35 or Cdk5 were generated by John Forsayeth and Steve Hardy and were used at a multiplicity of infection of 50 plaque forming units (pfu) per cell.

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